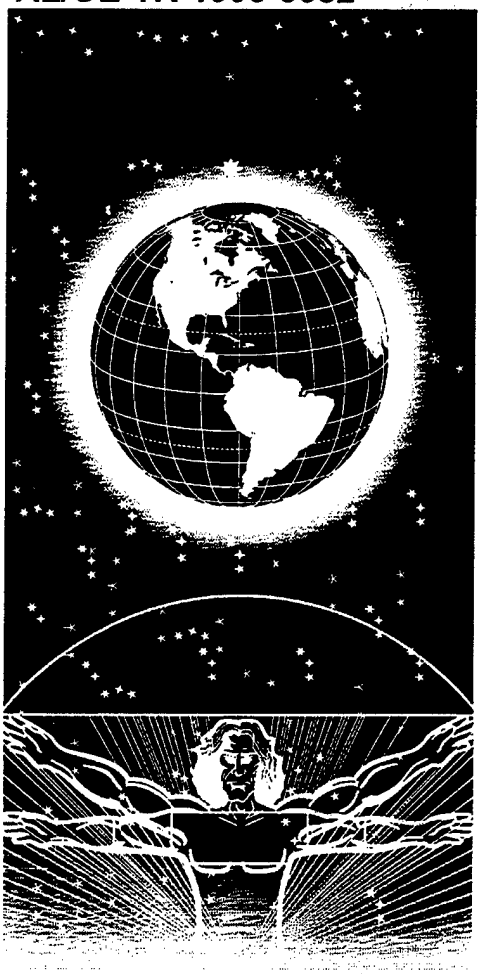
AL/OE-TR-1996-0082



UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

# USE OF ELECTRON PARAMAGNETIC RESONANCE IN OCCUPATIONAL AND ENVIRONMENTAL TOXICOLOGY

**Linda Steel-Goodwin**
OCCUPATIONAL AND ENVIRONMENTAL
HEALTH DIRECTORATE TOXICOLOGY DIVISION
ARMSTRONG LABORATORY
WRIGHT-PATTERSON AFB, OH 45433-7400

**Alasdair J. Carmichael**
ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
BETHESDA, MD 20991

**June 1996**

**Occupational and Environmental Health Directorate**
**Toxicology Division**
**2856 G Street**
**Wright-Patterson AFB OH 45433-7400**

## NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Air Force Armstrong Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Service
8725 John J. Kingman Rd., Ste 0944
Ft. Belvoir, Virginia 22060-6218

## DISCLAIMER

This Technical Report is published as received and has not
been edited by the Technical Editing Staff of the Air Force Armstrong Laboratory.

## TECHNICAL REVIEW AND APPROVAL

**AL/OE-TR-1996-0082**

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE DIRECTOR**

**STEPHEN R. CHANNEL**, Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Armstrong Laboratory

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | June 1996 | Interim Report - October 1993-May 1996 |

**4. TITLE AND SUBTITLE**
Use of Electron Paramagnetic Resonance in Occupational and Environmental Toxicology

**5. FUNDING NUMBERS**
Contract
PE 61102F
PR 2312
TA 2312A2
WU 2312A202

**6. AUTHOR(S)**
L. Steel-Goodwin, and A.J. Carmichael

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**
AL/OE-TR-1996-0082

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION AVAILABILITY STATEMENT**
Approved for public release; distribution is unlimited.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

The principles of experiments using the electron paramagnetic resonance technique tailored to the EPR/spin trapping and EPR/spin labeling studies of projects involving liver blood and testis is described. The methodology focused on quality control and sensitivity of the data measured by the EMS104 spectrometer. The hyperfine coupling constant of 13C-TCE after radicals were generated by 10Gy $\gamma$-irradiation is aN=1.42 mT, aBH=-.39 mT and a13C=0.78 mT. The principles described supplement free radical studies in Occupation and Environmental Health reported by OL/OE from 1993-1996.

| 14. SUBJECT TERMS | | | 15. NUMBER OF PAGES |
|---|---|---|---|
| Electron paramagnetic resonance | EPR | spin label | 42 |
| spin trapping | 13C-trichloroethylene | erythrocytes | 16. PRICE CODE |
| liver | testis | | |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| UNCLASSIFIED | UNCLASSIFIED | UNCLASSIFIED | UL |

# PREFACE

This is one of a series of technical reports originating from the electron paramagnetic resonance data generated for the Occupational and Environmental Health Directorate, Toxicology Division. This document serves as the interim report on radical analysis capabilites from October 1993 through May 1996. The report describes the principles used in performing electron paramagnetic analysis over the period October 1993 through May 1996. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division. The spectra shown in this report were sponsored by the U.S. Air Force Office of Scientific Research Environmental Initiative Program WORK UNIT 2312A202 under the direction of Dr. John Frazier, S&T. The authors wish to thank CPT C.R. Miller, MSC, USA and TSgt W.J. Schmidt, USAF for the human liver slices used in this study.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## ABBREVIATIONS

| | |
|---|---|
| 3-CAR | 2,2,5,5- Tetramethyl-1-pyrrolidinyloxyl-3-carboxyamide |
| d | Day |
| DBNBS | 3,5-dibromo-4-nitrosobenzene sulphonate |
| DMPO | 5,5 dimethyl-1-pyrroline-1-oxide |
| EPR | Electron Paramagnetic Resonance spectrometer |
| g | Gram |
| G | Gauss |
| kg | Kilogram |
| L | Liter |
| mg | Milligram |
| ml | Milliliter |
| mm | millimeter |
| N | number |
| p | Probability |
| PBN | N-tert-butyl-α-nitrone |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| Watt | W |

# SECTION I

# INTRODUCTION

The aim of this report is to serve as a tailored operator protocol for the EMS104 EPR analyzer to support *in vitro* liver slice analysis projects. The EMS104 is the first EPR designed to specifically quantify paramagnetic material. It serves as a quantitative tool for generation of predictive risk models for toxicology assessment of chemicals used in military acquisitions.

EPR spectroscopy and EPR/spin trapping are the most powerful techniques for detecting and characterizing free radicals (Buettner, 1987). EPR is a specific method because the only materials which exhibit EPR contain unpaired electrons. A free radical in this report is defined as an atom, molecule or compound with one or more unpaired electrons. Because free radicals are highly reactive one way of detecting them is by spin trapping. The reaction of a free radical with a spin trap, yields a more stable spin adduct which can be detected by EPR. Spin traps are usually nitrone or nitroso compounds and they can be used to study free radical pathways at the cellular and subcellular level. Free radical pathways are thought to play a major role in toxic mechanisms in various organs (gut, lung, heart, kidney, brain, liver), in the toxicity of various xenobiotic agents in these organs, and in vascular disorders (Carmichael et al., 1993a). The focus of free radical induced pathological conditions revolves around oxygen-related species (Steel-Goodwin et al., 1992). However, several important reactions involving active oxygen and nitrogen-centered radical species have also been investigated (Carmichael., et al., 1993b). Free radical decomposition products have been studied in operational chemicals to

develop mathematical models such as those performed by Bcyzkowski and Flemming, 1996 and to understand the effects of free radicals on intestine (Steel-Goodwin and Carmichael 1994), on liver cells (Steel-Goodwin et al., 1994) and testis cells (Wyman et al., 1996). More recently chemicals which can cause cancer have been studied in liver slices to evaluate the use of EPR for development of a mathematical description of both operational chemicals and chemicals occurring as environmental contaminants at military installations (Steel-Goodwin et al 1996a,b). Currently EPR/spin labeling techniques are being used to evaluate cell membrane lipids and cell receptor/protein interactions after ammonium dinitramide exposure (Steel-Goodwin et al., 1996 c,d). This compound is one of the candidates being considered by the USAF to replace ammonium perchlorate as a constituent of solid rocket fuel (Borman, 1994).

## SECTION 2

## METHODS AND RESULTS

*LEVEL OF UNDERSTANDING*: A research level knowledge of chemistry, physics, mathematics and statistical techniques is required for studies using electron paramagnetic resonance spectroscopy (EPR). The EPR technique is rarely used because of lack of expertise, facilities and the availability of other spectroscopic techniques. In compliance with Good Laboratory Practices, another technique as well as EPR, or EPR/spin trapping should be used to corroborate data. Suitable other techniques are magnetic resonance such as nuclear magnetic resonance (NMR), or electron nuclear double resonance (ENDOR) and GC/MS, HPLC, fluorometric or uv/vis spectrophotometric techniques.

***RESEARCH QUESTION:*** It is imperative to understand the research question or final goal in order to correctly apply EPR expertise to answer the question. This can be achieved if EPR technology is addressed up front and early and the EPR operator is admitted as an active participant of the ***planning*** of the experiments. Because EPR is not a commonly understood technique, normally a final draft only is provided. Regardless of the circumstances, the EPR operator must be provided sufficient information to document what possible knowledge the above mentioned confirmation techniques give when applied to the research question. This information should be supplied in memorandum format for placement in the laboratory notebook to serve as a record the research question was addressed for EPR technology and possible alternative approaches in the planning of the project.

*INITIAL EXPERIMENTS*: Wherever possible, perform pilot studies for feasibility. Any study of free radicals should be carried out initially without animal tissues to determine the

correct conditions and parameters for studies of the chemical. If at all possible the chemicals to which the samples were exposed should be known. All samples should be analyzed in the same manner once the procedure is established. However, remember if no results are obtained that does not equal that studies are not feasible. Once initial results are obtained, vary conditions in order to get a better handle on the system. **NEVER, NEVER PREDICT OUTCOME BASED ON INITIAL RESULTS.** This tends to confuse the issue and invariably leads the project down the wrong path. It also tends to interpretation of experimental results or bias in the context of what you want to see. **DO NOT DO THIS**. Results should be interpreted on the basis and context of what they are saying, regardless of what the project manager would like them to say. In initial experiments only make assumptions do not conclude.

***EXPERIMENTS:*** As more information is gathered after varying conditions on initial results and experiments, vary conditions at each step of the way when progress is made, or apply other necessary techniques to answer other questions.

***EPR INSTRUMENT***: The EMS104 analyzer was designed to be compact, portable and carry out routine analysis of samples. The magnet is encased and the manufacturer claims no microwave hazard to EPR operators. The EMS104 can analyze solid samples and liquid samples (20 to 50 ul volume). Samples are added to quartz glass sample tubes which are placed into the sample cavity of the instrument. Parameters are set by the computer so that manual setting of the machine is not required. As the equations below show, if there is no magnetic field, there will be no signal. Human errors in EPR operation such as failure to turn on the magnetic field are thus prevented. The limit of detection of the EMS104 is at least $2 \times 10^{10}$ spins/$10^{-4}$ T. It is linked to a microprocessor (XPS P90

Dell) via a RS232 interface which can be used to start remote acquisition and data collection for post processing using the WIN-EPR® 3.0 program (Bruker, Billerica, MS). This EPR data is interoperable with other EPR analyzers located at other military bases (e.g. ESP 300E at the Armed Forces Radiobiology Research Institute, Bethesda MD; ER200 Electron Nuclear Double Resonance Spectrometer at the Naval Research Laboratory, Washington DC and the ESP300E at the US Army Research Institute of Chemical Defense, Aberdeen Proving Ground, MD). The computer connection provides the capability for instantaneous communication across the information highway to verify data and to encourage networking and a corporate memory of unique EPR generated spectra between laboratories.

***SPECIMEN REQUIREMENTS:***. EPR is specific for detection of paramagnetic compounds. The technique is described for specimens which have been exposed to spin trap or spin label and which have been analyzed in the liquid or solid state. This technique can be used to detect radicals in biological tissues and cells. The EMS104 can also measure paramagnetic materials in fuels and clays. The procedure described below has proven successful with slices from liver and testis and erythrocytes(Steel-Goodwin and Hutchens, 1995, Steel-Goodwin et al., 1996 a,b,c,d and Wyman et al., 1996).

***SAMPLE PREPARATION:*** As with all projects, it is imperative that the EPR operator provide a description or training of what way samples should be prepared. This is the best way to provide service to those providing specimens. The EMS104 can only measure free radicals within its range of detectability, $2 \times 10^{10}$ spins/$10^{-4}$ T. Free radicals caused by toxic chemicals are famously difficult to detect because their intermediates are so reactive in biological systems they do not persist long enough to accumulate in sufficient

concentrations for appropriate detection spectroscopically. Radicals in specimens can be detected by reaction with spin traps, to form longer lived radicals, that are also paramagnetic and can be detected by EPR. Radicals can react on exposure to ultraviolet light so experiments were performed without the use of fluorescent lights. Also biological samples are prone to enzymatic reduction to diamagnetic products that cannot be detected by EPR. To prevent reduction, samples were immediately frozen and concentrated by lyophilization.

***PRINCIPLES OF THE ANALYTICAL PROCEDURE:*** The principal technique used to determine free radicals is electron paramagnetic resonance spectroscopy (EPR). EPR analysis requires samples with detectable concentrations of radicals. EPR is the most sensitive and direct method of measuring free radicals (Rice-Evans et al., 1991). In general, the technique measures the effect of a magnetic field on an unpaired electron (free radicals and transition metals). The spinning electron acts as a small magnet. The unpaired electron also interacts with neighboring nuclei. When placed in an external electric field, information is obtained regarding the local environment surrounding the unpaired electron. From quantum mechanics the most basic equations pertaining to EPR are:

**1.** $\Delta E = h\nu$

**2.** $E = g\,\mu_B B_0 M_S = +/- 1/2\, g\,\mu_B B_0$

**3.** $\Delta E = h\nu = g\,\mu_B B_0$

where $\Delta E$ is the energy difference associated with electromagnetic energy; h is Planck's constant; $\nu$ is the frequency of the radiation; $B_0$ is the magnetic field ; $\mu_B$ is $\beta$ or the Bohr magnetron, the natural unit of electronic moment; and g is the g-factor, which is a

proportionality constant approximately equal to 2 for most biological samples. From these equations the field of resonance $B_{res}$ (G) for the EMS104 spectrometer for a g=2 signal at a frequency of 9.5 GHz is 33.89 mT. Measurement of g-factors does not give information about the molecular structure of the sample. This information is provided by the interaction of the unpaired electron with neighboring nuclei. The nuclei of atoms in a molecule or complex often have a magnetic moment which produces a local magnetic field at the electron. The interaction between the electron and the nuclei is called the hyperfine interaction. This provides information on the identity and number of atoms that make up the molecule or complex as well as distances from the unpaired electron. Computer simulation packages are available to simulate the spectra and predict the structure of the radicals using the hyperfine coupling constants which can be measured from the spectra. In biological systems free radicals are mostly short-lived and highly reactive species reacting at diffusion controlled rates so spin trapping is used for their detection. Spin trapping consists of reacting a short lived radical with a spin trap, usually a nitrone or nitroso compound yielding a longer lived nitroxide spin adduct which can be detected by EPR (Buettner 1987).

***SPIN TRAPS:*** There are a number of spin traps which can be used to study free radicals at the cellular and sub-cellular level of tissue (Mason, 1984). The most common traps are nitrone or nitroso compounds. The structure of the most common spin traps is shown in Figure 1.

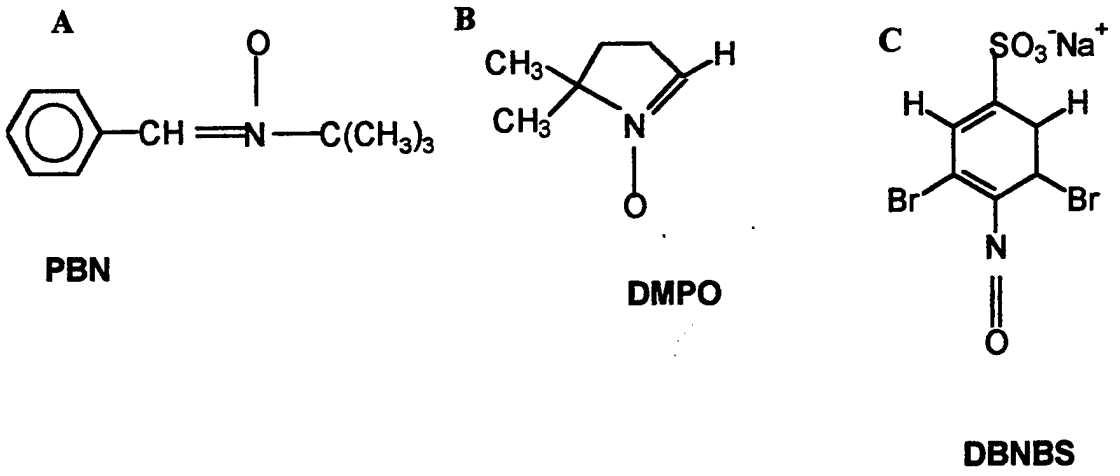



**Figure 1** ***The chemical structure of common spin traps.***

The nuclear spin of nitrogen is 1 so the spectrum produced will have 3 lines as shown in the equation below:

**4.** **$2I + 1$ = number of lines on the EPR spectra**

where I is the nuclear spin. The nuclear spin of common atoms are shown in Table 1.

| Atom | Nuclear Spin (I) | No. Of Lines |
|---|---|---|
| **Carbon** | **0** | **1** |
| **Oxygen** | **0** | **1** |
| **Nitrogen** | **1** | **3** |
| **Hydrogen** | **1/2** | **2** |

**Table I** ***Table of nuclear spins of carbon, hydrogen, nitrogen and hydrogen atoms.***

The concentration of the spin trap used in sample preparation is a trade-off between the solubility of the trap in aqueous media, the biological effect of the trap on mitochondria and the trapping efficiency for the radical of study. Once the parameters are established for a particular experiment the concentration should not be varied. For example, the spin trap N-tert-butyl-a-phenyl nitrone (PBN) and 3,5-dibromo-4-nitrobenzenesulphonate (DBNBS) can detect radicals in liver at a concentration of 10 mM. PBN did not change viability data from control samples (Steel-Goodwin et al 1996a) however 10 mM of DBNBS decreased viability (unpublished data).

***SPIN LABELS***: Spin labeling is a technique which makes use of stable nitroxide radicals to label biological components of a cell allowing them to be monitored by EPR. A nitroxide spin label can act as a biological marker and yield information on the environment and motion of the component to which it is attached. Subtle changes in environment and motion of the spin labeled component are observed and measured through changes in the nitroxide EPR line shape. Nitroxide spin labels have been used to measure radiation and chemical effects on cell membranes (Steel-Goodwin et al 1996c). Using the EMS104, spin labels were used to study changes in the nitroxide EPR line shape when the label was lyophilized with mouse liver slices with and without the nitroxide spin trap PBN (Steel-Goodwin and Hutchens, 1995) and also to measure cell membrane fluidity and protein receptor binding (Steel-Goodwin et al 1996c,d).

***INSTRUMENT PROCEDURES:*** Operation of the instrument for analysis of liver slice samples involves successful completion of a number of tasks which are described below.

These procedures should be followed as stated. Further information can be obtained in the instrument manual (Bruker, 1994) or by calling for technical support from the vendor. ***PREVENTATIVE MAINTAINANCE:*** The EMS 104 is computer driven. Preventative maintenance involves protection of the instrument from dust and moisture. Dust and moisture in the cavity can interfere with the signal generated. Gold, used in construction of the EMS104 is sensitive to dust. The instrument is supplied with plastic covers to protect it from dust and moisture. When the instrument is turned off and not in use it should be covered. The instrument requires 20 minutes to equilibrate. During this *warm up* the cavity should not be exposed to air. A plastic plug is provided to cover the cavity at night and when the instrument has no sample in the cavity. Also do not permit other personnel to place papers, laboratory notebooks on top of the machine near the cavity when a sample is in the cavity. The sample tube is made of quartz glass which is very thin walled and easily broken. For liquid samples capillary tubes should be used. Many clays contain Mn and it is recommended the tube be plugged with Critoseal ® for aqueous samples. A special capillary tube holder is also available for ease of operation and analysis. The height of the sample can be determined using the sample gauge. The height of the sample platform can be raised or lowered by editing the parameters screen during initial set up. Should a sample tube break in the instrument, turn off the machine, unplug it from its connections and turn it upside down. It requires two personnel to turn the machine over as it weighs approximately 45 Kg. **Do not drop**. The instrument contains a ruby crystal which can be broken if the machine is dropped. The instrument is portable but requires careful packing for shipment on long journeys, especially by air freight. Loose glass will fall out when the instrument is turned over. Clean up the area and plug

the instrument back into its connections and recalibrate it. If the calibration is accepted, all the glass has been removed and moisture is not a problem. If the machine does not calibrate let it air for 1-8h and try the calibration again. If it still does not work, the cavity requires cleaning and possible replacement. This should be completed at the factory. Do not remove the cavity for shipment until requested to do so by the company.

***FUNCTION VERIFICATION*** The EMS104 is computer driven. When the instrument is turned on the computer boots its operating procedures and loads the EMS104 software. The software then initializes the microwave attenuator, the sample positioning device and the microwave oscillator and displays the main menu with the commands:

**SETUP CALIBRATE ACQUIRE PARAMETERS MANIPULATE RESULTS FILE HELP**

and the results window shows the system is uncalibrated by the display **!RESULTS.**

***STANDARDIZATION PROCEDURE*** The first procedure to perform after turning the instrument on is the instrument calibration. Press CALIBRATE. The display will show:

**[ESC] SAMPLE INSTRUMENT HELP**

Press INSTRUMENT. The computer loads the calibration software parameters, Table 2. Follow the commands on the screen. The calibrator for the EMS 104 is a quartz sample tube containing pitch. Clean the quartz tube with Kleenex to remove any powder or grease from the tube from gloves. Remove the sample plug and add the sample holder. Place the tube in the cavity and hand tighten the sample holder. Make sure the calibrator tube is added the same way each time with sample number facing straight forward.

| Parameter | |
|---|---|
| Power (mW) | 12.6 |
| Sweep Width (G) | 100 |
| Modulation (G) | 8.02 |
| Sweep Time (s) | 10.5 |
| Filter T.C. (ms) | 20.5 |
| Receiver Gain (dB) | 30 |
| Receiver Offset | 0 |
| Receiver Phase | 0 |
| Field Offset (G) | 0 |
| Number of Sweeps | 1 |
| Sample Height (mm) | 0 |

**Table 2** ***Parameters for instrument calibration***

The spectrum observed should be similar to the one below, Figure 2.

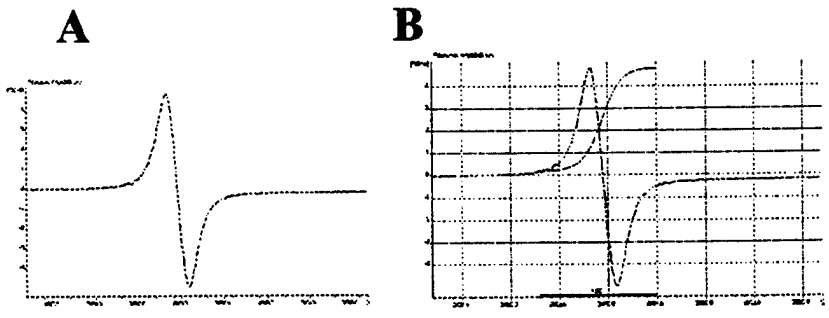



**Figure 2** **(A)** ***Spectrum of pitch*** **(B)** ***Double integration of pitch***

The report generated by analysis of the sample using the computer program WIN/EPR (Bruker Instruments, Billerica, MS) gives the value of the double integration of the spectrum.

Integral List Date: 29.04.1996 Time: 13:28
File Name : A:\pit245.spc
Normalisation value: N = 6.632e+004

| Start[] | End[] | DI | DI/N |
|---|---|---|---|
| 3457.2727 | 3487.2825 | 3.078e+010 | 4.641e+005 |

**Figure 3** ***Display of double integration of spectrum of instrument calibrator.***

***METHODOLOGY:***

1. ***Manufacturer's Standard***

Once the instrument has been calibrated PRESS ACQUIRE. This will acquire a spectrum of the calibrator and the data will be printed on the printer paper and computer screen. Perform this 10 times and record the value. The data shown in the Figure 2 A below has a mean ± SD of 999.61 ± 1.11.

**A**

| Run # | Reading |
|---|---|
| 1 | 1000.6000 |
| 2 | 1001.9000 |
| 3 | 999.2000 |
| 4 | 1000.1000 |
| 5 | 999.1000 |
| 6 | 999.3000 |
| 7 | 1000.1000 |
| 8 | 998.5000 |
| 9 | 999.3000 |
| 10 | 998.0000 |
| mean | 999.6100 |
| SD | 1.1130 |
| SE | 0.3520 |

**B**

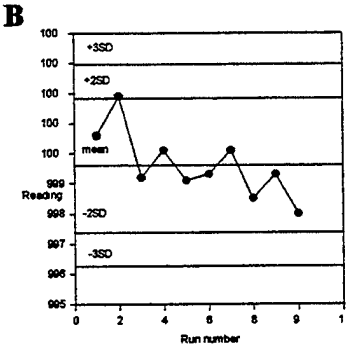



**Figure 4** ***Data of 10 consecutive EPR sample runs of pitch. (A) EPR readings. (B) Plot of EPR readings.***

The instrument software sets the pitch sample at 1000 by measuring the pitch sample shifts and trends in the instrument function over time can be recorded. A downward trend is shown in Figure 4B when the mean ± 2 SD and ± 3 SD, of 10 consecutive runs of pitch were plotted. A trend or shift occurs when there is a change in data that persists for more than five independent analytical runs. In this example the downward trend was corrected by performing another instrument calibration. When 20 data points are obtained a QC-chart can be created using these data.

Variance can occur as humidity in the laboratory varies due to weather conditions but also in response to electromagnetic waves emitted from certain military equipment (such as radar or surveillance equipment). During these conditions do not operate the machine. Each calibration of pitch is kept within the memory of the computer and can be accessed by pressing CONTROL C. If the instrument calibration shows no shifts or trends then biological samples can be run.

2. ***Reagent, standard, control, and materials:***

a) ***Reagents:*** The same media used to perform experiments in tissues must be used to prepare standards and controls. For liver and testes slices the reagent media is Waymouth's (Gibco). The Waymouth's is stable for one week. Waymouth's medium was supplemented with 10 mM HEPES, 5 mM NaCl, 15.5 mM $NaHCO_3$, 2.4 mM L-glutamine(Gibco), 50 mg/ml gentamycin sulfate (Gibco), and 10% w/v fetal bovine serum (Gibco).

b) ***Standards and controls:*** The spin label 3-CAR is used for the making standards. Known concentrations of 3-CAR are added to the Waymouth's media containing liver slices. A stock solution of $1 \times 10^{-4}$ M of 3-CAR was prepared. Known

volumes are added to Waymouth's media containing liver slices in pre-weighed scintillation vials. The standard mixture is homogenized for the same time as specimens. The mixture is frozen in liquid nitrogen and lyophilized for 18 h. The weight of the lyophilized samples are recorded.

c) Biological samples are treated the same way as standards and controls.

d) Weigh standards, controls and samples into glass capillary tubes. These are inserted into quartz glass EPR tubes (Wilmad Glass EPR tubes 706-PQ 950) and inserted into the cavity of the EMS104 EPR analyzer and an EPR spectrum generated.

3. For analysis of lyophilized liver slices press parameters in the EMS104 main menu and edit set the parameters as shown in Table 2.

| Parameter | |
|---|---|
| Power (mW) | 25.06 |
| Sweep Width (G) | 100 |
| Modulation (G) | 4.02 |
| Sweep Time (s) | 10.5 |
| Filter T.C. (ms) | 20.5 |
| Receiver Gain (dB) | 60 |
| Receiver Offset | 0 |
| Receiver Phase | 0 |
| Field Offset (G) | 0 |
| Number of Sweeps | 1 |
| Sample Height (mm) | 0 |

**Table II.** ***Parameters for analysis of lyophilized liver samples.***

4. Make a directory and run the sample calibration curve. Run the first control spectrum. Store this spectrum in the directory and run the first sample. Continue until all controls and samples are measured. Each sample should be run in duplicate. Twenty

samples plus controls can be run per day. The quantitative data can be printed out and affixed to the laboratory notebook.

***DATA REDUCTION*** The calibration curve for each assay is stored in the permanent memory in the EMS 104 Analyzer. The stored curve is generated by assaying samples with increasing concentrations of the spin label. A non-linear least-squares regression calculates the best fit curve using the spin label calibrator samples, one of which is zero. Curve parameters of slope, span of magnetic field, the value between the high and low calibrators are used to determine the best fit. Concentrations of samples in unknown specimens are read from this curve using the EPR readings generated for each sample on the standard run. The specimens are normalized for sample weight. All samples are measured on a Mettler balance using standard weights to verify balance accuracy. Data is calculated using the Sigma Plot and the total sample data reported. All calculations are placed in the laboratory notebook including the linear calibration curve for each assay run.

***DATA STORAGE***. During the working period of the project the data should also be stored on the EMS104. A directory should be made for each assay run. Each sample should be stored as a filename within the directory. A hard copy of the quantitative printout should be secured in laboratory notebook. On completion of the project the data should be removed from the EMS104 and archived. The EMS104 data for archiving can be stored on a 31/4 diskette and on the hard drive of the Dell microprocessor in a directory. The data can be transferred electronically using the following steps:

1. Turn on the EMS 104.
2. Turn on the Dell microprocessor on.

3. Go to the WINEPR program on the Dell microprocessor.

4. Open the EPR icon.

5. Press CONTROL 'e' until you get a $ prompt.

6. Type DIR.

7 Type chd ems104

8 Check all directories

9 For example Type chd KIDNEY

10 Type C: KERMIT

11 At the Kermit prompt type SEND *.*

12 Type send _D *.*

13 Go to transfer and do receive by escaping back into the local system and pressing the receive prompt. NOTE: If there are too many letters in the filename it will not transfer.

***METHOD LINEARITY*** The machine can detect 2 X $10^{10}$ spins/$10^{-4}$ T. Each study can be tailored to provide a standard for the research sample. At least four standards should be prepared in the same way as the assay calibrators and samples. This is an advantage when compared to backup Varian E9 which requires manual adjustment of instrumentation parameters for each reading as well as manual analysis of the spectrum.

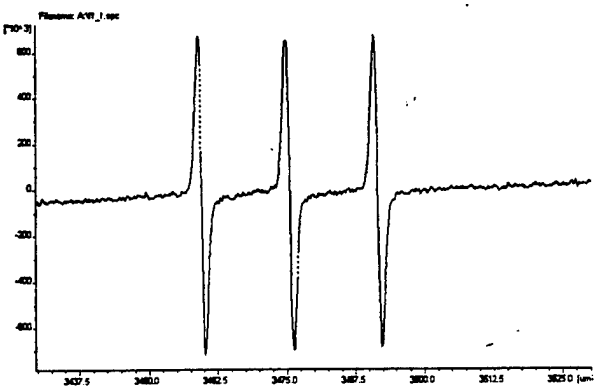
***METHOD SENSITIVITY*** The lowest 3-CAR sample used in the standard curve for an experiment should be set as the low limit of the 3-CAR.

***CONTROL DATA CRITERIA*** Plot the control pitch data on the appropriate chart. The range of the controls is initially set by the manufacturer at 1000. When 20 data points are obtained a QC chart is created using these data.

***SPECIFICITY:*** The EMS104 only measures paramagnetic material therefore it is specific.

***EPR SPECTRUM***: The typical EPR spectrum of 3-CAR in water is shown in Figure 5A.

**A**



**B**

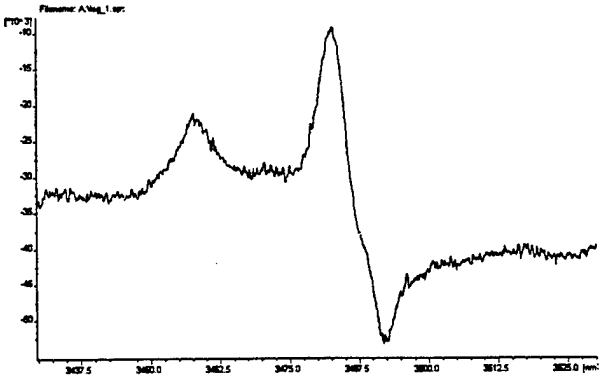


**Figure 5** ***(A) 3-CAR in liver homogenate. (B) Lyophilized sample of A.***

This spectrum may be measured for quantitative data or qualitatively. This is a spin label or a stable free radical. In solution with liver homogenate it spins freely. The sample, 20ul, was placed in a capillary tube (Curtain Matheson Scientific), which was sealed with Critoseal® and placed in an EPR capillary tube holder (Bruker Instruments, Billerica, MS). When the sample is lyophilized the spin label is immobilised giving the spectrum,

Figure 5B. Lyophilized spectra were used to quantitate radicals in liver slices (Steel-Goodwin et al 1996a,b). Note the parameters used to measured the spectra for the liquid sample and solid samples were the same. Liquid samples are used to measure hyperfine coupling constants.

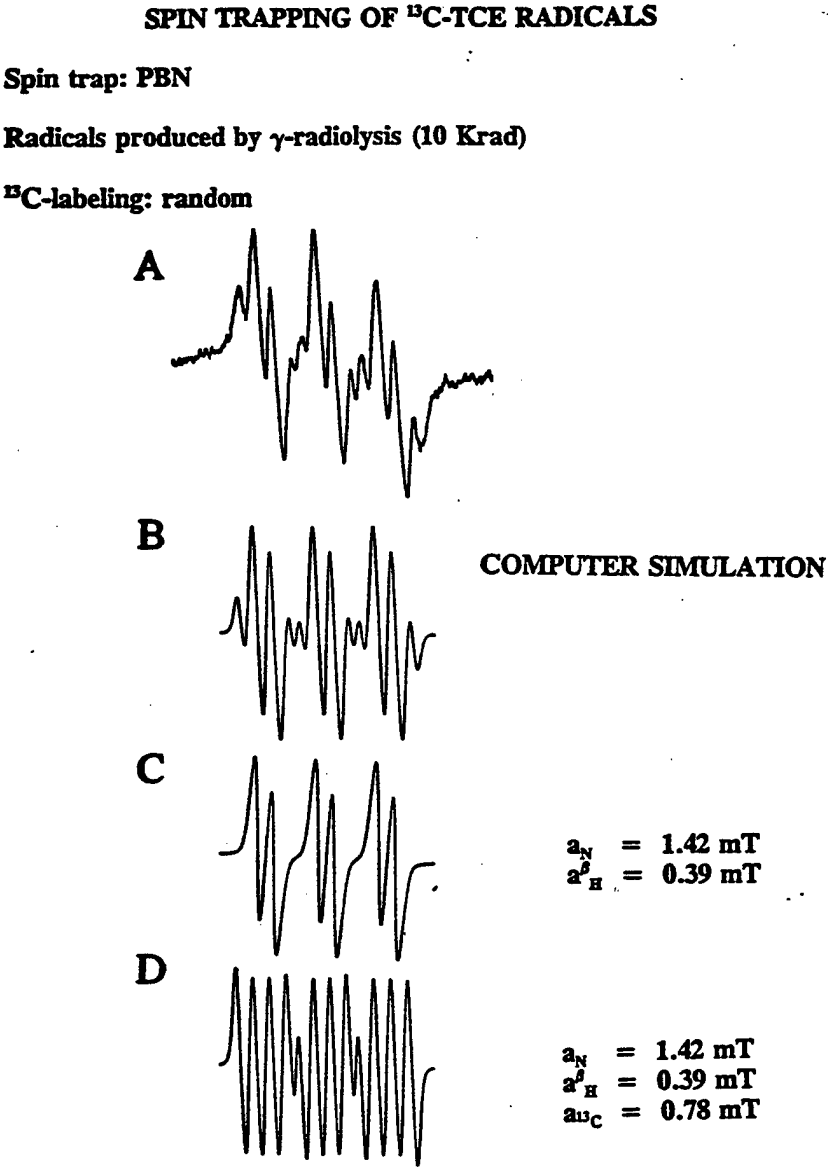



**Figure 6** ***(a) Spectrum of $^{13}$C-TCE radical (b) Computer simulated spectrum of $^{12}$C-TCE radical (c) $^{13}$C-TCE radical (d) Computer simulated $^{13}$C-TCE radical***

Spin trapping of $^{13}$C-TCE radical adducts of PBN produced by γ-irradiation of the sample with a total dose 10Gy produced by a $^{60}$Co γ-ray source is shown in Figure 6. The hyperfine coupling constant of the $^{12}$C-TCE is $a_N$ = 1.42 mT and $a^{\beta}_H$ = 0.39 mT. The $^{12}$C-TCE radical is described elsewhere (Steel-Goodwin and Carmichael 1995). The $^{13}$C-TCE has an extra spin so the hyperfine coupling constant is $a_N$ =1.42 mT, $a^{\beta}_H$ = 0.39 mT, and $a^{13}C$ = 0.78 mT. The use of a non-radioactive isotope confirmed the structure of the TCE radical described by Steel-Goodwin and Carmichael, 1995.

***CALIBRATION CURVE:*** Using the embedded calibration curve program in the machine a calibration is performed with each sample run using at least 5 controls. From this data regression analysis is performed (Steel-Goodwin and Hutchens, 1995). Based on the curve the results of unknown samples can be calculated.

***PEAK HEIGHT ANALYSIS*** Data is analyzed on the EMS104 using the peak-peak analysis.

***DOUBLE INTEGRATION***: Double integration analysis should be performed using the WINEPR program (Bruker). Do not use the EMS104 unless the spectrum is a single peak. Biological samples do not normally produce a single peak. To transfer the spectrum to the WINEPR use the Kermit command or save the data on a diskette and manually load it onto the computer.

***CORRELATION OF PEAK HEIGHT AND DOUBLE INTEGRATION:*** A study was performed to check the correlation between peak height measured on the EMS104 and the and double integration data measured using the WINEPR program. The two were proportional (Steel-Goodwin and Hutchens 1995).

*QUALITY CONTROL* The concepts of quality assurance must be fully understood and correctly applied to meet the objectives of the Quality Control (QC) program. The data from a quality control specimen can lie within the two standard deviation limit and the data may still be "out -of-control". Shifts, trends or changes in sample distribution data can represent out-of-control situations which are of as great concern as control data that exceed the established standard deviation range. The policy is to investigate any shift or trend in data that persists for more than five independent analytical runs. It must be emphasized that there is absolutely no substitute for sound judgment based on an appreciation of the analytical system, the technique, the quality control materials utilized and the analytical interpretation of the data generated by the procedure. Each spectrum of the EPR is an independent run as the computer performs a check of all parameters using the ACCESS mode.

*SAFETY PRECAUTION SYNOPSIS* Handling biological specimens always involves the risk of infection. In the worst cases this could be Hepatitis or AIDS. Human and animal tissue is submitted for analysis so all specimens and blood controls must be handled as if capable of transmitting these diseases. Wear gloves and disinfect all tools (pipets, EPR sample holders etc) and the work area with 70% ethanol or 3% Chlorox when finished.

**HAZARDOUS REAGENTS**: The MSDS of all reagents used in the analysis should be read to determine proper safety precautions, storage and disposal. All hazardous chemicals should be disposed of as described by the MSDS and established laboratory procedures.

*SPECIAL NOTES:*

1. Before preparing standards and controls remove them from the refrigerator and allow them to warm up to ambient temperature before use. This prevents errors in results from volume/pipetting errors. All prepared samples and specimens should be stored in the dark in a decissator.

2 A. The first report should be made once sufficient experiments are done to have an understanding of the system. To address the issue of how much testing is enough, reports that provide information that can be taken as facts or which are statistically sure, should be written up. However, those results which are weak require reassessment to see what else is required to make them sure.

2 B. When preparing a quantitative EPR report, give the data of instrument calibrator as well as the radical data determined. When experiments are all analyzed bring back the original question to see what the results answered in the context of the question. Report the facts of how these results *within the original question* were addressed and how these results fit within the context of the original question. Only allow a minimal amount of speculation to show the work breakdown structure. If results are to be part of a scientific paper the following guidelines are additionally recommended:

1. Always write a paper as if you are telling a story
2. Take writing as an extension of yourself
3. For scientists writing is the ultimate expression of:

   EYE, MIND AND HAND COORDINATION

4. EYE-HAND COORDINATION: How you did your experiments and what you observed.

5. Mind-Hand Coordination: How you interpreted what you observed and explain it in writing.

6. The simpliest explanation that makes sense usually means a better understanding of the problem. Therefore, the best approach is a straight forward approach.

7. If you have to hedge, speculate or find your way around an issue it usually means you need much more experiments to draw a conclusion.

2 C. The following is also a guide of the layout of an EPR paper:

1. Bring out the original question

2. If the original question was answered go to 4.

3. If the original question was not answered, but another important point was **state it.**

4. Lay out possible figures which explain what was answered or which explain the points addressed.

5. Write possible equations and mechanisms.

6 WRITE INTRODUCTION

a. Give background knowledge.

b. State the objective.

c. State the results.

7. Write experimental procedures.

8. With the layout of figures and equations in 4 and 5 explain each one in an orderly fashion.

Arroyo et al., (1993) is a good example of the use of this guidance. The first figure included the initial experiment and initial result. From this result as many conclusions were drawn as possible. As many possible conclusions were eliminated by explanation in writing and in the context of what is known in the literature. The ones that could not be eliminated were addressed by further experiments. That's where subsequent figures entered the picture. Each figure was explained in sequence and the final conclusion written.

2 D. Data generated by the EMS104/ WINEPR can be transferred through the electronic highway and stored in spreadsheets and bitmaps or directly pasted into word documents. Computer assistance permits data to be stored in the form of an electronic laboratory notebook if desired to reduce paper copies.

# SECTION 3

# DISCUSSION

Experiments referenced have been performed using a Varian E109 (Carmichael et al 1993a,b) or a Bruker EMS 300E EPR spectrometer (Steel-Goodwin et al., 1994, Steel-Goodwin and Carmichael, 1995). These EPRs are located at another military base and required travel. Recent experiments have been performed using the in-house EMS 104 EPR Analyzer (Steel-Goodwin and Hutchens 1995, Steel-Goodwin et al 1996b, Wyman et al., 1996). All spectra shown in this report were stored on the EMS104 and electronically transferred to the word processor for data handling. The backup machine for the EMS104 is a Varian E4 EPR. During this study period the EMS 104 there were no instrument problems. In the example of the pitch calibrator, Figure 2-4 , the mean ± SD was 999.6 ± 1.11. This example showed a downward trend, Figure 4B which was corrected by recalibrating the instrument.

The EMS104 has been used for one, quantitation of chemical induced free radicals, two, quantitation of alterations of membrane fluidity and three, protein receptor binding estimation. The EMS104 EPR technique is specific for paramagnetic materials and does not affect the measured sample physically or chemically. Thus, it can yield both qualitative information (ie whether or not a sample has detectable radicals) as well as quantitative results (the concentration of radicals compared to a standard).

There are a number of spin traps and spin labels which are commercially available for studying free radicals (Mason, 1984). Selection of the spin trap depends on the properties of the radical and the biological system under study. For liver and testis slices

the spin traps N-*tert*-butyl-α-nitrone (PBN) has yielded detectable signals (Steel-Goodwin and Hutchens, 1995, Steel-Goodwin et al., 1996a,b and Wyman et al., 1996). Numerous spin traps (Mason, 1984 and Buettner, 1987) can be used in cells and subcellular fractions. There are numerous commercially available spin labels and the doxyl spin label has proven useful not only for quantitation of unknown radicals but in structural studies of cells such as WB344 cells and erythrocytes (Steel-Goodwin et al 1996c,d). Parameters for the use of 3-CAR for creation of a calibration curve are given. Finally, to identify a radical it is usual to use a non-radioactively labeled custom made isotope of the chemical under study. The structure of the radical is determined by comparison of the radicals detected by a spin trap with the labeled isotope and the unlabeled chemical of interest. Hyperfine coupling constants can be measured directly from the spectra. This data can be used to computer simulate the spectra and thus predict the structure of the radical. For example, the $^{13}C$-TCE radical is shown in Figure 6. This is the radical generated after γ-irradiation. Any substance which is irradiated forms free radicals which are detectable by EPR. Oxygen free radicals are continuously being produced intracellularly e.g. by mitochondrial oxidation. Under physiological conditions there is a fine balance between the production of free radicals and their removal by antioxidant defense systems. The term oxidative stress is used when the balance is disrupted e.g. when antioxidant systems fail to scavenge free radicals produced from TCE exposure. Catalytic transitional metal ions like copper and iron play a major role in free radical formation and trigger the conversion of less reactive species e.g. hydrogen peroxide. In tissue injury there will be increased metal ion availablity and an acceleration of free radical reactions. The leakage of metal ions after tissue damage amplifies the initial tissue insult. For this reason the effects of radicals on

the transport of iron was studied (Steel-Goodwin et al., 1996c,d). Based on EPR studies (Steel-Goodwin et al., 1996 a,-d) with liver, testes and erythrocytes, it can be expected that mitochondria of cells exposed to radical forming chemicals would be the targets of radical damage and this should be determined by histology.

# SECTION 4

# REFERENCES


**Arroyo, C.M., F. Feliciano, D.L. Kolb, J.R. Keeler, S.R. Millette and R.R. Stotts** (1993) Autoionization reaction of phosgene (OCCl2) studied by EPR. J. Biochem Toxicology 8: 107.

**Borman, S** (1994) Advanced energetic materials emerge for military and space applications. Chemical and Engineering News 72: 18.

**Bruker Analytiche Messtechnik** (1994) EMS104 User's Manual, Bruker Instruments, Billericia, MS.

**Buettner, GR.** (1987) Spin trapping: ESP parameters of spin traps. Free Rad Biol & Med (3) 259.

**Byckowski, J.Z., and C.D. Flemming** (1996) Computer-aided dose-response characteristics of chemically initiated oxidative stress in vitro The Tox 30 (1) A1227.

**Carmichael A.J., L. Steel-Goodwin, B. Gray, and C.M. Arroyo** (1993a) Reactions of active oxygen and nitrogen species studied by EPR and Spin trapping. Free Rad Res Comms 1993: (19) S1.

**Carmichael A.J., L. Steel-Goodwin, B.Gray and C.M. Arroyo** (1993b) Nitric oxide interaction with lactoferrin and its production by macrophage cells studied by EPR andspin trapping. Free Rad. Res. Comms (19) S201

**Carmichael AJ and L. Steel-Goodwin** (1994) Decomposition of Peroxynitrite Studied by EPR/Spin Trapping Free Rad Biol Med (2) 3 Q 5.

**Mason, R.P.** (1984) Spin trapping free radical metabolites of toxic chemicals. In Spin Labeling in Pharmacology Chapter 2 pp87-129.

**Rice-Evans, C.A., A.T. Diplock, and M.C.R. Symons** (1991) Techniques in free radical research. Editors R.H. Burdon and P.H. van Knippenberg, Elsevier, NY.

**Steel-Goodwin L, C.M. Arroyo, B.H. Gray and A.J. Carmichael** (1992) EPR detection of nitric oxide dependent spin adducts in mouse jejunum. In Biology of Nitric Oxide Vol. 2 Enzymology, Biochemistry and Immunology, ED S. Moncada, MA Marletta, JB Hibbs Jr and EA Higgs Portland Press, London pp 80-84.

**Steel-Goodwin L, and A.J. Carmichael** (1994) Nitric oxide and smooth muscle relaxation in the intestine. Chemical and Radiation effects measured by EPR/spin trapping. CRC Press Chapter 7 In Radiation and the Gastrointestinal tract A. Dubois, GL King and DR Livengood Eds.

**Steel-Goodwin L, T.L. Pravecek and A.J. Carmichael** (1994) Trichloroethylene: An EPR Study Free Rad Biol Med: (2) 3 Q10.

**Steel-Goodwin, L., and B. Hutchens** (1995) Efficiency of PBN to trap 3-CAR in B6C3F1 mouse liver slices: An EPR study AL/OE-TR-1995-0139.

**Steel-Goodwin, L., T.L. Pravecek, B. Hancock, W.J. Schmidt, S. Trivunovic, G. Miller, C. Bishop, W. Batholomew, S.R. Channel and A.J. Carmichael** (1996a) Trichloroethylene: Free radical studies in B6C3F1 mouse liver slices. AL/OE-TR-1996.

**Steel-Goodwin, L., W.J. Schmidt, C.R. Miller, J.Z. Bycokowski, and A.J. Carmichael** (1996b) Quantitation of free radicals in B6C3F1 mouse liver slices on exposure to four chemical carcinogens: An EPR/Spin trapping study. AL/OE-TR-1996.

**Steel-Goodwin, L., K.W. Dean, C.D. Flemming, F.C. Music, and A.J. Carmichael** (1996c) Uses of EPR/spin labeling as a biomarker. AL/OE-TR-1996.

**Steel-Goodwin L, K.W. Dean, S.Berty, J. Armengol. A. Young, M.D. Pace and A.J. Carmichael** (1996d) Potential of electron paramagnetic resonance techniques to assess ammonium dinitramide exposure. Submitted for publication.

**Wyman, J.F., L. Steel-Goodwin, and A.J. Carmichael** (1996) Free radicals in precision cut bovine testicle slices: Effects of 1,3,5 trinitrobenzene measured by electron paramagnetic resonance AL/OE-TR-1996.